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Antiphospholipid antibodies (APA) and recurrent pregnancy loss: treating a unique APA positive population*

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BACKGROUND: Recurrent pregnancy loss (RPL) has been associated with antiphospholipid antibodies (APA) including anticardiolipin and lupus anticoagulant. Therapy using heparin and aspirin has been shown to significantly improve the live birth rate. We evaluated whether other APA should be considered as a basis for treatment in women with RPL. We also assessed the efficacy of heparin and aspirin therapy compared with aspirin alone in these women. METHODS: A two-centred, prospective, cohort evaluation of 79 women with two or more consecutive pregnancy losses who underwent a complete evaluation for RPL that was negative except for positive APA. Women with RPL and APA to cardiolipin (CL), phosphatidyl serine (PS) and/or lupus anticoagulant (LAC) treated with heparin and aspirin (group 1) were compared with those with other positive APA (to phosphatidyl inositol, phosphatidyl glycerol and/or phosphatidyl ethanolamine) treated with heparin or aspirin (group 2) or treated with aspirin alone (group 3). RESULTS: There were no significant differences in patients' demographics between groups. There were 19 viable infants born to 25 women (76%) in group 1, 18 viable infants born to the 28 women (64%) in group 2, and 12 viable infants born to the 26 women (46%) in group 3. Only the comparison between group 1 and group 3 reached statistical significance ($P = 0.03$). CONCLUSION: APA other than CL, PS and LAC may be associated with RPL.

Key words: anticardiolipin antibodies/antiphospholipid antibodies/aspirin/heparin/recurrent pregnancy loss

Introduction

Antiphospholipid antibodies (APA) are a group of autoantibodies that bind to negatively charged phospholipids. These antibodies have been associated with thrombotic events which could lead to pregnancy loss (Lockshin, 1997). Clinical features such as venous or arterial thrombosis, unexplained fetal deaths after 10 weeks, delivery at <34 weeks as a result of severe pregnancy-induced hypertension (PIH) or recurrent pregnancy loss (RPL) before 10 weeks in combination with a positive laboratory result of antibodies to cardiolipin (CL) or a positive lupus anticoagulant (LAC) on two occasions at least 6 weeks apart are parameters for diagnosis of the antiphospholipid antibody syndrome (APS) (Wilson *et al*., 2001). A recent review has highlighted the pathophysiologies, clinical manifestations, and management guidelines related to APA (Kutteh *et al*., 1997).

Low dose aspirin, heparin, prednisone and i.v. immunoglobulin have been proposed as treatments for APS with mechanisms that affect both the immune and coagulation system to counteract the effects of APA. Theoretical mechanisms for APA-induced thrombosis include decreased prostacyclin production by endothelial cells, increased thromboxane production by platelets, and decreased protein-C activation (Chamley *et al*., 1993; Shibata *et al*., 1993). Exogenous heparin has been shown to inhibit the binding of APA *in vitro* in a dose–response manner; thus, endogenous heparin produced by trophoblasts may function in a similar fashion (Ermel *et al*., 1995). The antithromboxane effects of aspirin on inhibition of platelet aggregation are thought to work in concert with heparin to promote and enhance implantation (Patrono, 1994; Hauth, 1995). Prednisone and i.v. immunoglobulin have been proposed to alter the immune system and autoantibody action (Spinnato *et al*., 1995), while low dose aspirin may improve placental blood flow by decreasing the thromboxane to prostacyclin ratio, thus enhancing implantation (Rubinstein *et al*., 1999). Currently, all of these therapies are being utilized as treatments for women with RPL and APA.

In prospective studies, the use of s.c. heparin and aspirin has resulted in successful deliveries in ~75% cases of women with RPL and APA with a low frequency of obstetric and maternal complications (Kutteh, 1996; Rai *et al*., 1997). However, these reports included women who were positive for the common phospholipids (CL, PS and LAC). We identified a

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group of women with a history of RPL and other positive APA and compared their obstetric history to women with RPL and CL, PS or LAC. We prospectively followed these women to determine if a positive test for antibodies to phosphatidyl inositol (PI), phosphatidyl glycerol (PG), and/or phosphatidyl ethanolamine (PE) would suggest a need for treatment with heparin and aspirin or if aspirin therapy alone might offer them an improved live birth rate.

Materials and methods

Subjects

More than 1400 women were evaluated for RPL and APS at the Southwestern Fertility Associates of The University of Texas Southwestern Medical Center in Dallas and at the University Fertility Associates of The University of Tennessee at Memphis between July 1993 and July 2000. Women eligible for this study included those who desired to have a pregnancy, agreed to have a complete evaluation, had at least two consecutive pregnancy losses, had positive APA and/or LAC on at least two occasions, and agreed to the treatment protocol. The American College of Obstetricians and Gynecologists has recently stated that the causes of RPL are similar in women who have suffered two losses when compared with women who have had three losses. 'Thus, patients with two or more consecutive spontaneous abortions are candidates for an evaluation to determine the aetiology, if any, for their recurrent pregnancy loss' (Carson and Branch, 2001). The study groups were reviewed and selected based on a complete, normal evaluation for RPL and a positive test for IgG and/or IgM antibodies to phospholipids on two separate occasions. Women were categorized as those with RPL and the common APA (CL, PS or LAC) and women with RPL and other APA (PI, PG or PE). These women were treated as follows: CL, PS and/or LAC treated with heparin and aspirin (group 1); PI, PG and/or PE treated with heparin and aspirin (group 2); or PI, PG and/or PE treated with aspirin alone (group 3). Women were excluded from this study if they had an allergy to aspirin, a combination of antibodies between group 1 and group 2 or between group 1 and group 3, a documented bone disorder, or another abnormal finding from the evaluation.

The diagnosis of APS was based on a well-documented history of RPL (at least two spontaneous, consecutive miscarriages fathered by the same partner) and positive levels of immunoglobulin (Ig)M or IgG APA ($\geqslant$20 phospholipid units) on two separate occasions at least 6 weeks apart. Each woman had a complete evaluation for RPL, which included a history and physical examination, karyotypes on both partners, hysterosalpingogram or hysteroscopy, mid-luteal progesterone, thyroid stimulating hormone, prolactin, lupus anticoagulant [partial thromboplastin time (aPTT), dilute Russell's viper venom time (dRVVT), and platelet neutralization test], IgG and IgM APA, and cervical cultures for mycoplasma, ureaplasma and chlamydia. All women who participated were started on low dose aspirin (81 mg/day) and prenatal vitamins prior to conception. The initial dosage of heparin was 5000 IU s.c. twice daily if the woman's weight was $>$150 lbs and 6000 IU twice daily if her weight was $<$150 lbs. All patients were instructed to have quantitative β-hCG and progesterone level with any missed period and continued aspirin (81 mg/day) at the first confirmed pregnancy test. Those women with progesterone levels to $<$20 ng/dl were treated with vaginal progesterone 50 mg twice daily until 10 gestational weeks. For those women utilizing heparin, twice daily s.c. injections of heparin (5000–6000 IU) were started at the first confirmed pregnancy test. All patients were counselled, given the same information concerning treatment options, and were given literature on the risks and benefits of heparin. Those patients who did not desire heparin were advised to take low dose aspirin (81 mg/day) throughout pregnancy (group 3). The Institutional Review Boards of The University of Texas Southwestern Medical Center and University of Tennessee Memphis Health and Science Center approved the treatment protocols.

APA enzyme-linked immunosorbent assay (ELISA)

All serum samples were evaluated for IgG and IgM antibodies against CL, PS, PI, PG, and PE utilizing the ELISA method (Harris, 1990). Briefly, individual 96-well microtitre plates (Immulon-2; Dynatech Labs, Chantilly, VA, USA) were coated with 30 µl of either purified phospholipids (Sigma Chemical Co., St Louis, MO, USA) at a concentration of 45 µg/ml (CL) in ethanol or 50 µg/ml PI, PS, PG and PE in methanol. The plates were air-dried overnight at 4°C then blocked with 200 µl of 10% fetal calf serum (FCS; Gibco, Long Island, NY, USA) in 1×phosphate-buffered saline (PBS; Gibco), washed and incubated at 37°C for 2 h with 50 µl of patients' sera diluted 1:50 in 10% FCS in PBS. Each unknown sample was run in duplicate. The plates were then washed to remove unbound antibody and proteins, and a secondary antibody, alkaline phosphatase-conjugated antihuman IgG (Caltag Labs, San Francisco, CA, USA) or IgM (Biosourse; Tago Immunologicals, Camarillo, CA, USA) was added to the plate. After incubation and washing, *p*-nitrophenyl phosphate substrate (Sigma 104; Sigma Chemical Co.) was added and used to indirectly measure the level of APA in a patient's serum. The optical density of the samples, caused by the cleavage of the substrate by the enzyme, was determined at 405 nm by a Bio-Tek Microplate Reader Model EL 340 (Bio-Tek Instruments, Winooski, VT, USA) and was used to quantify the amount of APA in the sera.

Every assay plate also included a known high positive anticardiolipin sample [$>$100 GPL (IgG phospholipid units)] run in duplicate. Plates were incubated until the high positive wells achieved an optical density of $>$1.0; typically, this required an incubation of 20–30 min. Referenced standard sets for cardiolipin (Louisville APL Diagnostics, Inc., Atlanta, GA, USA) and known negative sera were used on every plate. All results were defined in phospholipid units for IgG (GPL) and IgM (MPL) as follows: $<$10 IU, negative; 10–19 IU, borderline; 20–80 IU, positive; and $>$80 IU, high positive. PS, PI, PG and PE values were interpreted based on the multiples of the median (MoM) method as described previously (Kutteh *et al*., 1994; Branch *et al*., 1997). Known positive samples with PI, PG, PS and PE were included in every plate.

Briefly, phospholipid units for IgG and IgM were calculated for each serum sample, and the median value was determined from the non-Gaussian distribution. The cut-off value in phospholipid units of each APA was determined by using the 99th percentile of the normal population, ~3.0 times the median value (Kutteh *et al*., 1994). All values reported as positive were the mean of duplicated determinations with background absorbance obtained from wells prepared without the coating phospholipid subtracted. Any values with SE $>$10% were discarded and reassayed. Inter-assay variation was $<$8%, and intra-assay variation was $<$6%.

Heparin and aspirin treatment

Women with RPL and APA who desired treatment initiated treatment with s.c. heparin (5000–6000 IU) every 12 h with the first positive pregnancy test. Platelets and PTT were obtained 6 h after heparin injection weekly for 2 weeks after the initiation of heparin therapy and 1 week following any adjustment in heparin dosage. Thereafter, platelets and PTT were checked periodically to ascertain that they were in the normal range. The heparin dosage was adjusted downward if the PTT was elevated outside the normal range or if the platelet count fell to $<1\times10^5$/ml. Supplementation with calcium carbonate

to achieve a total daily intake of 1.5 g/day was prescribed for all patients, an approach that has been reported to counteract the osteoporotic effects of heparin (Dahlman *et al*., 1994).

Each pregnancy was documented by transvaginal ultrasonography scheduled at 7 weeks gestation for the determination of fetal heart motion. Additional ultrasonography was performed as indicated, but generally baseline sonograms were obtained at 20 weeks. Antenatal testing (fetal kick counts, non-stress tests, or biophysical profiles) was initiated at 28–30 weeks when indicated. Aspirin was discontinued 2 weeks before the estimated due date. Heparin was continued until full term and was discontinued when the patient initiated spontaneous labour. The evening heparin dose was omitted prior to planned amniocentesis or a scheduled operative delivery.

Statistical analyses and data collection

Statistical analyses were performed using one-way analysis of variance. A comparison of all pairs of columns was performed using Tukey–Kramer multiple comparison test. A post-hoc analysis was performed if $P < 0.05$. A power analysis was performed with the following assumptions based on previous data (Kutteh, 1996): the data were sampled from populations with identical SD; the data were sampled from populations that follow Gaussian distributions; expected delivery of live births in group 3 (aspirin alone) = 0.40; expected delivery rate in group 1 and group 2 (heparin and aspirin) = 0.75; significance $\alpha = 0.05$; and $\beta = 0.80$. For a one-tailed analysis, the number of patients required in each group to detect a 35% difference in a successful delivery was 18 women in each group.

Each patient was personally interviewed and medical records were reviewed to confirm pregnancy histories. Outcome data included maternal and obstetric complications. Preterm birth included deliveries before 37 completed menstrual weeks. Gestational diabetes included women who demonstrated glucose intolerance requiring dietary or medical control. Minor bleeding included haematuria, nosebleeds, gum bleeds, and bleeding at the injection site. Thrombocytopenia was defined as platelet counts $<1\times10^5$/ml. Pre-eclampsia was diagnosed based on hypertension and proteinuria. Intrauterine growth retardation (IUGR) was diagnosed when birthweight was below the 10th percentile for gestational age. Major bleeding included abruption, blood losses at delivery requiring transfusion, or vaginal bleeds requiring hospitalization during pregnancy. Karyotype analysis was recommended on all subsequent miscarriages; however, this was not always possible.

Results

Demographics

There were no significant differences in the patient's age at entry, prior pregnancies, prior live births, prior miscarriages, gestational age at the time of loss, or the number of losses that occurred before 12 weeks gestation between groups (Table I). More than 90% of the miscarriages in all three groups occurred before 12 weeks gestation. There were no statistical differences in the proportion of women that were positive for both IgG/IgM isotypes compared with isolated IgG and IgM isotypes. The isolated IgG antibodies were more prevalent for all groups compared with isolated IgM antibodies (Table I).

Obstetric outcome

As shown in Table IIA, there were no significant differences in viable infants born to patients who received heparin and aspirin in group 1 (19/25; 76%) versus group 2 (18/28; 64%). Thus, the women with RPL and the common APA (group 1) and the women with RPL and other APA (group 2) had a similar outcome after treatment with heparin and aspirin. There was a very slight trend toward significance ($P = 0.20$) in the live birth rate between group 2 (18/28; 64%) and group 3 (12/26; 46%) and a significant difference between groups 1 and 3 ($P = 0.03$). There were no differences in the average

Table I. Comparison of recurrent pregnancy loss study patients

	Group 1 (n = 25)	Group 2 (n = 28)	Group 3 (n = 26)
Age at entry (years)	33.2 ± 3.9	33.0 ± 4.4	33.6 ± 4.6
Total pregnancies	4.2 ± 1.6	4.3 ± 1.3	4.8 ± 1.8
Prior live births	0.5 ± 0.7	0.6 ± 0.4	0.6 ± 0.3
Prior miscarriages	3.6 ± 1.0	3.7 ± 1.0	4.3 ± 2.0
95% CI	(3.2–4.0)	(3.3–4.1)	(3.7–5.1)
No. of patients with			
2 losses (%)	3 (12)	4 (14)	3 (12)
>2 losses (%)	22 (88)	24 (86)	23 (88)
EGA at loss (weeks)	9.1 ± 4.9	8.9 ± 3.2	7.9 ± 2.7
Losses <12 weeks (%)	92.9	96.4	96.2
Isotypes of APA			
IgG antibodies only (%)	8 (32.0)	11 (39.3)	10 (38.5)
IgM antibodies only (%)	3 (12.0)	4 (14.3)	6 (23.1)
IgG and IgM antibodies (%)	14 (56.0)	13 (46.4)	10 (38.5)

Values are mean ± SD, except where stated otherwise. No significant differences between groups.
95% CI = 95% confidence interval; EGA = estimated gestational age.

Table IIA. Outcome data from antiphospholipid antibody patients who had live births

	Group 1	Group 2	Group 3	P
Live births (%)	19/25 (76)	18/28 (64)	12/26 (46)	[a]
95% CI	(59–95)	(48–80)	(34–66)	
EGA at birth (weeks)	37.7 ± 1.6	37.8 ± 2.2	38.1 ± 1.6	NS
Birthweight (g)	3192 ± 448	3308 ± 296	3214 ± 266	NS
Vaginal delivery	13/19 (68)	14/18 (78)	8/12 (75)	NS

[a]Group 1 versus group 3 ($P = 0.03$); group 1 versus group 2 ($P = 0.28$); group 2 versus group 3 ($P = 0.20$).
95% CI = 95% confidence interval; EGA = estimated gestational age; NS = not significant.

Table IIB. Outcome data from recurrent pregnancy loss patients with antiphospholipid antibodies who had miscarriages

	Group 1	Group 2	Group 3	P
Miscarriages (%)	6/25 (24)	10/28 (36)	14/26 (54)	[a]
EGA at loss (weeks)	10.4 ± 3.9	9.7 ± 3.2	9.1 ± 2.3	NS
Blighted ovum	1/6 (17)	2/10 (20)	4/14 (29)	NS
No fetal heart motion	1/6 (17)	3/10 (30)	3/14 (21)	NS
Abnormal karyotype[b]	1/6 (17)	2/10 (20)	2/14 (14)	NS

[a]Group 1 versus group 3 ($P = 0.03$); group 1 versus group 2 ($P = 0.28$); group 2 versus group 3 ($P = 0.20$).
[b]Karyotype analysis was recommended on all products of conception. Data were available from three women in group 1, six women in group 2, and seven women in group 3.
EGA = estimated gestational age; NS = not significant.

Table III. Obstetric and maternal complications of patients who delivered a liveborn

Complication	Group 1 (n = 19)	Group 2 (n = 18)	Group 3 (n = 12)
Obstetric			
Preterm birth	2 (10.5)	1 (5.5)	0 (0)
IUGR	2 (10.5)	1 (5.5)	1 (8.3)
Maternal			
Gestational diabetes	2 (10.5)	0 (0)	1 (8.3)
Minor bleeding	3 (15.8)	2 (11.1)	2 (16.7)
Thrombocytopenia	0 (0)	0 (0)	0 (0)
Pre-eclampsia	2 (10.5)	0 (0)	1 (8.3)
Major bleeding	0 (0)	0 (0)	0 (0)
Fractures	0 (0)	0 (0)	0 (0)

No significant differences between groups (values in parentheses are percentages).
IUGR = intrauterine growth retardation.

birthweight of infants or the frequency of vaginal deliveries among groups.

As shown in Table IIB, there were six miscarriages in group 1 (24%), 10 miscarriages in group 2 (36%) and 14 miscarriages in group 3 (54%). The miscarriages in all groups occurred on average between 8 and 11 weeks. Seven of the patients who miscarried had a blighted ovum. A total of seven miscarriages were documented with no fetal heart motion. Data from the karyotype analysis on products of conception were available from three women in group 1, six women in group 2, and seven women in group 3. There were no differences in the frequency of abnormal karyotypes between groups (Table IIB).

Heparin use during pregnancy

All women in group 1 and group 2 with RPL were treated with heparin and aspirin throughout pregnancy. Women in group 1 and group 2 initiated heparin treatment by 5.2 ± 1.0 and 5.1 ± 1.0 gestational weeks and continued treatment for an additional total of 32.5 ± 2.0 and 32.9 ± 1.8 weeks prior to delivery respectively. Women in group 3 initiated aspirin (81 mg/day) prior to conception and continued until 2 weeks before the estimated due date.

Complications of treatment

Documented obstetric and maternal complications were low (Table III). There were no significant differences in preterm birth and IUGR among groups. None of the patients had thrombocytopenia, major bleeding, or fractures. In the women who had a subsequent pregnancy loss while being treated with heparin and aspirin, there were no apparent differences in the identifiable causes of pregnancy loss.

Discussion

The data presented in this study identified a population of women with other APA (n = 54) as having similar prior risk for a miscarriage as those women with positive CL, PS and/or LAC antibodies. The demographics of women in all three groups were compared and evaluated. There were no significant differences in the demographics of women with RPL who were found to have any of the APA. These observations suggest that the women with other positive APA (groups 2 and 3) may have experienced an obstetric course similar to those women in group 1. Positive APA were of the IgG-only isotype in 29/79 women (25%) who tested positive compared with 14/79 women (18%) with the IgM-only isotype. We did not evaluate IgA APA in this study, because IgA CL have not been recognized as significant by the international consensus report (Wilson *et al*., 2001). The clinical significance, if any, for isolated IgA-only antibodies is unclear at this time and must be interpreted with caution (Peaceman *et al*., 1993; Carreras *et al*., 2000; Alfirevic *et al*., 2002). However, continued reports of IgA APA underscore the need for careful interpretation of positive results and correlation with the obstetric history when considering treatment options.

The lack of standardization among APA laboratories has made it difficult for physicians to identify patients with APS and those at risk for a miscarriage (Peaceman *et al*., 1993; Kutteh *et al*., 1994; Carreras *et al*., 2000). Thus, an international group of investigators has established both clinical and laboratory criteria for the diagnosis of APS (Wilson *et al*., 2001). Yet, problems still exist when pregnancy loss patients are referred to infertility clinics that may have had testing performed at different laboratories using different control values and cut-off values to determine positive results. Also, standard testing may exclude a population of APA patients who have significant obstetric problems but test positive for other APA and negative for the most common antiphospholipids such as CL or LAC.

This ongoing debate of the clinical significance of CL, LAC and other APA has prompted some investigations to screen patients using a panel of APA for the evaluation of RPL. For example, Branch *et al*. analysed the 95th and 99th percentile as the positive and negative cut-off for a panel of phospholipids among 147 women with RPL, APS and fertile controls (Branch *et al*., 1997). By using the 99th percentile, they found that 26/147 (17.7%) had positive antibodies to CL and 13/147 women (8.8%) with RPL demonstrated binding against phospholipids other than CL or LAC. Based on comparison with controls, they concluded that this difference was not clinically significant. In a much larger study, the prevalence of APA among 866 women with RPL was investigated. In this population, 17% of women had a positive level for CL antibodies in comparison with 10% of women with antibodies other than CL (Yetman and Kutteh, 1996). Although this study was retrospective, it suggests that 10% of women with RPL would not have been identified for a risk of APA if diagnosis was based on a test for CL and/or PS and/or LAC antibodies exclusively.

This study also evaluated heparin and aspirin therapy and aspirin therapy alone to treat a population of women who may have gone untreated, if APA other than CL, PS and/or LAC were excluded as risk factors for an adverse pregnancy outcome. We previously reported that women with RPL and CL antibodies demonstrated a successful delivery rate of 44% with aspirin therapy alone compared with 80% heparin and

aspirin therapy (Kutteh, 1996). Based on these data, assumptions could be made for the expected delivery rate among treatment groups which were used to estimate the sample size.

For this study, we compared the pregnancy outcomes of women who had a normal evaluation except for positive CL, PS and/or LAC treated with heparin and aspirin (group 1); positive PI, PG and/or PE antibodies treated with heparin and aspirin (group 2); and positive PI, PG and/or PE treated with aspirin alone (group 3). The live birth rate was 76% for group 1, 64% for group 2 and 46% for group 3. These data are in agreement with prospective reports that have shown a successful obstetric outcome with heparin and aspirin therapy and/or aspirin alone therapy in women with positive CL, PS and/or LAC antibodies (Cowchock *et al*., 1992; Kutteh, 1996; Yetman and Kutteh, 1996; Rai *et al*., 1997; Empson *et al*., 2002). As expected, women in group 1 (RPL with common APA) treated with heparin and aspirin had a successful outcome in the majority of cases. The women in group 2 (RPL with other APA) had a live birth rate that was not significantly different from group 1 ($P = 0.28$). Successful deliveries occurred significantly more often when comparing group 1 with group 3 ($P = 0.03$), but not when comparing group 2 with group 3 ($P = 0.20$). While it is inconclusive from this study which treatment should be considered for those women with RPL and antibodies to PI, PG and/or PE, there was a trend toward improved outcome in the group treated with heparin and aspirin. A larger, prospective, randomized, controlled trial is necessary to elucidate this question.

The significance of a panel of APA to diagnose APS is an ongoing debate with many complex questions that can only be addressed with larger APA study groups. We were able to identify 54 women with a history of RPL and positive APA other than CL, PS or LAC, who had an otherwise normal evaluation. This required screening of 1400 couples over 7 years and suggested a possible diagnosis in ~4% of the women tested. Although there are different views on the necessity for clinical testing of PG, PI, and PE for the diagnosis of APS, it appears that, in 8–10% of women with RPL, identification of these other APA may suggest a direction for treatment (Yetman and Kutteh, 1996; Branch *et al*., 1997). This study is limited to a small but significant study group of 54 women (groups 2 and 3) with RPL and other APA who may benefit from additional treatment. A larger group of women treated prospectively in a randomized study is necessary to ascertain the clinical significance of the individual phospholipids PI, PG and PE.

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